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Spatially-Explicit Assessments of Genetic Biodiversity and Dispersal in Gopher Tortoises for Evaluation of Habitat Fragmentation at DoD Sites

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Christopher W. Theodorakis
Southern Illinois University

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14. ABSTRACT
The objective was to perform a "proof of principle" assessment of genetic diversity and gene flow of gopher tortoises on Camp Shelby, MS. The specific aims were to determine if there is evidence that military activity affects genetic diversity and gene flow. DNA was extracted from the blood of gopher tortoises collected from 22 colonies in and around Camp Shelby. The amount of genetic diversity in each colony and treatment group was determined for microsatellite DNA markers and mitochondrial DNA. The data indicate that the amount of genetic diversity may be affected by a combination of military activity and habitat quality. Data on gene flow indicate that military activity may have affected the rates of dispersal among the tortoise colonies. Genetic distances did not correlate well with geographic distance. This may indicate that gene flow is affected by factors other than geographic distance in these tortoises, possible by military activities on Camp Shelby. Overall, the data indicate that there is enough genetic diversity among these colonies to use genetic approaches in delimiting critical habitat needs for conservation of gopher tortoises, which was the main goal of the research.

15. SUBJECT TERMS
Gopher tortoise, conservation genetics, microsatellite, mitochondrial DNA, Army, Camp Shelby, Mississippi, geneic diversity, dispersal, gene flow

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LIST OF ACRONYMS

DNA – Deoxyribonucleic Acid
DoD – Department of Defense
DOE – Department of Energy
F_{ST} – Fixation index
GIS – Geographic Information Systems
MCMC – Markov Chain Monte Carlo
mtDNA - Mitochondrial DNA
PCR – Polymerase Chain Reaction
SERDP – Strategic Environmental Research and Development Program
SIUE – Southern Illinois University Edwardsville
SON – Statement of Need
TES – Threatened and endangered species
URTD - Upper Respiratory Tract Disease
USDA – United States Department of Agriculture

LIST OF FIGURES AND TABLES

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1.0 EXECUTIVE SUMMARY

Gopher tortoises (*Gopherus polyphemus*) have declined to as little as 20% of their original abundance. This is *significant* because this tortoise is a keystone species in the longleaf pine forest ecosystem of the southeastern US. Habitat destruction and fragmentation have contributed significantly to the tortoises' decline. Because of habitat fragmentation, and because they live in colonial burrows, gopher tortoise populations exist as scattered metapopulations in a heterogeneous landscape. Sustainability of metapopulations depends upon sufficient opportunities for dispersal among populations. To this end, genetic analyses could be used to identify patterns of gene flow among colonies or groups of colonies, potential source/sink dynamics, and potential dispersal corridors necessary for sustaining metapopulations and habitats that are critical foci of genetic biodiversity and are a priority for conservation. However, there is little information on the patterns of genetic diversity and gene flow in the western part of the range of the gopher tortoise, where they are federally endangered.

Also, a large proportion of the remaining populations of this species reside on Department of Defense (DoD) installations. Thus, military facilities may be crucial for conserving the remaining populations. However, military activities could be compromised by land-use restrictions designed to protect habitat for species listed as threatened and endangered, such as gopher tortoises. Such conflicts may be ameliorated by proactive management plans that identify effects of habitat fragmentation or other anthropogenic disturbances (such as contamination), as well as identification of critical TES habitat on lands surrounding DoD facilities. In order to be effective and cost effective, such management plans must consider metapopulation processes (for example, dispersal and extinction/recolonization among a network of interconnected (sub) populations), which are essential for sustaining populations in fragmented habitats. However, assessing long-term, large-scale metapopulation processes using conventional methods – for example, mark recapture or telemetry – would be costly, labor-intensive, or infeasible in many cases [Reed et al. 2002]. An alternative approach would be to use population genetic and phylogeographic analyses to assess patterns of dispersal among scattered populations or sub-populations. However, this approach has not been investigated for many species on DoD lands. There is no information on gopher tortoise genetic diversity or gene flow in DoD installations and surrounding lands. This project is significant, because it provides data that is instrumental to filling these data gaps.

Therefore the *objective* was to perform a "proof of principle" assessment of genetic diversity and gene flow of gopher tortoises on Camp Shelby, MS. The rationale is that these data could be used to develop a project plan for a follow-on, multi-year study of the metapopulation genetics of these species in relationship to landscape structure, population demographics, and fitness components. This information could be used to identify habitat offsite of the DoD facilities that is critical conservation of these species by preserving genetic biodiversity and corridors of dispersal. Therefore, the *overall goal* was to determine if there was enough genetic diversity within and between colonies to perform these types of analyses. The *specific aims* were to determine if

1) The population on Camp Shelby was spatially structured.

2) The levels of genetic diversity and gene flow were affected by military activity and habitat quality.

3) If the patterns of genetic diversity followed an "isolation by distance" model – i.e., if there was a correlation between geographic distance and genetic relatedness.

For these purposes, DNA was extracted from the blood of gopher tortoises collected from 22 colonies in and around Camp Shelby. The amount of genetic diversity in each colony and treatment group was determined for microsatellite DNA markers and mitochondrial DNA. The amount of genetic diversity in mitochondrial DNA was analyzed in two different loci: a single-base sequence polymorphism in the cytochrome b gene (an enzyme in the electron transport chain) and the control region, a non-coding region that is the origin of DNA replication when the cell divides and replicates both nuclear and mitochondrial DNA.

Specific Aim 1: Is the population on Camp Shelby spatially structured?

Wright's fixation index F_{ST} was used to assess the amount of differentiation between-populations. If the Fst is not statistically significantly different from 0, then this does not support the hypothesis that the two populations being compared are genetically distinct. It was found that many of the Fst comparisons are not statistically significant. However, this is not surprising, given the small number of samples. Nonetheless, there were a number of significant Fst values, most of which indicate moderate differentiation among at least some of the colonies. Thus, the tortoises cannot be considered to be one homogenous, genetically indistinct population, because there is some spatial structuring.

Specific Aim 2: Are the levels of genetic diversity and gene flow affected by military activity and habitat quality?

Genetic diversity estimates showed different effects for microsatellite, mitochondrial cytochrome b markers, and mitochondrial control region markers. The microsatellite DNA and mitochondrial control region markers indicated that the colonies without military activity tended to have the least amount of genetic diversity, and colonies with military activity tended to have more genetic diversity. The mitochondrial cytochrome b sequences show a slightly different pattern of diversity. In this case, the colonies in the poor habitats tended to have the most diversity. These differences may be at least partially explained by the mutation and evolution rates of the markers. Because the cytochrome b gene codes for an important protein, its mutation rate is more constrained, and so may reflect more historical processes. On the other hand, the microsatellite and control region loci are not coding genes, so they may mutate and evolve more rapidly. Therefore, these loci may be affected more by recent processes.

Gene flow among colonies was determined by using genetic distance estimates to construct "dendrograms" or "evolutionary trees" of the populations. These dendrograms are pictorial representations of the genetic relatedness between populations. However, the dendrograms for the microsatellite markers and mitochondrial control region did not conform to what was expected on the basis of geographic distance. Geographically, the "no military activity" sites are clustered into two groups: one group contains Site 1, Site 2, and the State Land colonies in close proximity, and another group contains T44E and T44W (a tortoise refuge on Camp Shelby). The markers showed that the no military activity sites they are closer to the high-impact sites than they are to each other. Hence, it seems that military activity may have affected the rates of dispersal to or from the military sites.

Thus, it appears as though the levels of genetic diversity within colonies, and the amount of gene flow among colonies may be affected by military activity and habitat quality, although the specific patterns may be influenced by evolutionary rates of the markers and by patterns of inheritance.

Specific Aim 3: Do the patterns of genetic diversity follow an "isolation by distance" model?

This question was answered by performing Mantel tests. This analysis basically tests the correlation between geographic distance and genetic distance (the opposite of genetic

relatedness). If there is a positive correlation, then genetic distance increases (average relatedness decreases) as geographic distance increases – which is what is predicted by the isolation by distance model. However, genetic distance did not correlate well with geographic distance. This may indicate that gene flow is affected by factors other than geographic distance in these tortoises, possible by military activities on Camp Shelby.

Generally, differences between microsatellite and mitochondrial DNA, and among mitochondrial DNA loci can be due to a number of factors. First, microsatellite DNA is bi-parentally inherited, while mitochondrial DNA is maternally inherited. Consequently, microsatellite DNA gene flow is affected by movement of both males and females, while mitochondrial DNA only reflects female gene flow. Second, the control region evolves at a greater rate than the cytochrome b gene. Thus, the patterns revealed by the cytochrome b gene may reflect historical processes, while those revealed by the control region may be influenced by more recent events.

Overall, the data indicate that there is indeed enough genetic diversity among these colonies to use genetic approaches in delimiting critical habitat needs for conservation of gopher tortoises, which meets the stated goal of the research.

In general, the main goal of the project was met: it was ascertained that the gopher tortoises in an around a military base have enough genetic diversity to be useful in identifying parcels of land for acquisition. Therefore, future studies will include the following: 1) In order to estimate background genetic variation within and between colonies in the vicinity of Camp Shelby, individuals will be collected from state parks and national forests in and around Camp Shelby. Analysis of genetic diversity and gene flow will be determined for these colonies. Also, in order to determine the applicability of these approaches on a wider scale, genetic diversity will be determined for tortoises residing on Eglin Air Force Base, FL, and on the Savannah River Site (part of the eastern population of gopher tortoises). Gene flow and genetic diversity will be determined for gopher tortoise colonies in and around these facilities. 2) In order to increase the resolution and statistical power of the genetic analyses, more microsatellite loci will be sampled. Because Luikart and Cornuet [1998] suggested that up to 20 polymorphic markers may be needed to detect recent population bottlenecks, I will attempt to identify at least nine new polymorphic markers, either by adapting them from published work with other chelonian species [Scwartz and Karl 2005], or by *de novo* cloning and sequencing. In addition, more detailed analyses will also be carried out. Genetic methods could include Maximum Likelihood- or Bayesian Analysis-based tests. 3) The effect of landscape structure on genetic diversity and gene flow will be determined from aerial photographs and GIS analyses. The effect of landscape on genetic diversity and gene flow will be tested by comparing the level of genetic diversity (heterozygosity for microsatellites, nucleotide diversity for mitochondrial DNA,) and gene flow between areas with different landscape properties on Camp Shelby, Eglin Air Force Base, Savannah River Site, and the lands surronding these facilities.

2.0 OBJECTIVES

The objective of this project was to perform a 1-year preliminary assessment of genetic diversity and gene flow of gopher tortoises (*Gopherus polyphemus*) n Camp Shelby, MS, which would provide the basis for a spatially-explicit, GIS-based analysis of genetic diversity. This research is relevant to SERDP SON Number CSSON-06-01, which describes a need "to develop methods to identify the most ecologically important land parcels on and in the vicinity of DoD installations for which land protection could provide long-term species conservation benefits and avoid additional military training restrictions." [SERDP 2004]. Identification of populations

that are major sources of genetic diversity would meet that need. Another stated need is to "provide optimal habitat". Quantitative relationships between landscape/landuse and genetic endpoints, and identification of habitats that maximize desirable endpoints such as effective population size, genetic diversity, and emigration/immigration ratios would aid in identifying critical habitat. A third component of the SON is to "provide key linkages to other habitat". Analysis of the patterns of gene flow among gopher tortoise colonies combined with GIS-based landscape analysis would identify and characterize the connectivity among these colonies.

3.0 BACKGROUND

3.1 CAMP SHELBY

Camp Shelby, MS, is the largest state-owned US military training site in the United States [Pike 2008]. It encompasses more than 525 km^2 (52,500 ha) in portions of Perry and Forrest Counties, MS. Camp Shelby was established in 1915 and has been in continuous operation for training activities of the Army Reserve and Active Components of the Army, Navy, Marine Corps, and Air Force. "It is a training ground for the Abrams M1 Tank, Paladin Howitzers and home to the 3rd Brigade 87th Division Training Support", including artillery and infantry field training maneuvers [Pike 2008]. It is located in southern Mississippi approximately 19 km south of Hattiesburg. The facility contains a 15 km^2 "impact area" used for artillery training activities. Activity within this area includes "approximately 150 troop-firings per day, and the range-firing list includes M1A1 tanks, Bradleys (M2A3 and M3A3 Bradley Fighting Vehicles), self-propelled and towed artillery, mortars, laser-guided weapons, and small arms." [Pike 2008]. There are numerous gopher tortoise colonies on the base, which have been and are being monitored by the Nature Conservancy [Epperson and Heise 2003]. Various portions of the camp were closed and reopened several times from 1918-1956, but in 1956, Camp Shelby was designed as a Permanent Training Site, and Congress allocated money for the first permanent barracks in 1958. The present-day footprint of Camp Shelby was approved by the Army in 1959, and construction of the present-day landscape architecture commenced shortly thereafter [Pike 2008].

3.2 THE GOPHER TORTOISE

The gopher tortoise (family Testudinidae) is the only North American tortoise found east of the Mississippi River, resides in the southeastern coastal states from eastern Louisiana to southern South Carolina (Figure 1). They are divided into a western and eastern population by the Mobile and Tombigbee Rivers in Alabama (Figure 1). The western population is federally-listed as endangered, and they are federally- and state-listed as endangered, threatened, vulnerable, or a species of concern throughout the entire range [Wilson et al. 1997]. Gopher tortoises have a relatively long life span, with animals generally reaching 40-60 years of age [Ernst et al. 1994]. Sexual maturity is reached between nine and 20 years of age, depending on habitat [Epperson and Heise 2003]. Females lay one clutch of eggs per year, ranging from five to eight eggs, but may lay no eggs in some years [Epperson and Heise 2003]. It has been estimated that 80% of the original population has been lost in the last 100 years [Affenberg and Franz 1982]. The main threat is habitat destruction, modification, and fragmentation [Deimer 1992]. Other serious threats include predation and epizootics of upper respirator tract disease (URTD) – a diseases caused by *Mycoplasma agasazzi* [Deimer 1992,Brown et al. 1999]. Anthropogenically-derived predation threats include harvesting by people, and predation by introduced fire ants, coyotes (*Canis latrans*) and nine-banded armadillos [Deimer 1992, Main et

al. 2000]; the latter two species have expanded their natural range into the range of the gopher tortoise as a result of anthropogenic landscape modification [Main et al. 2000].

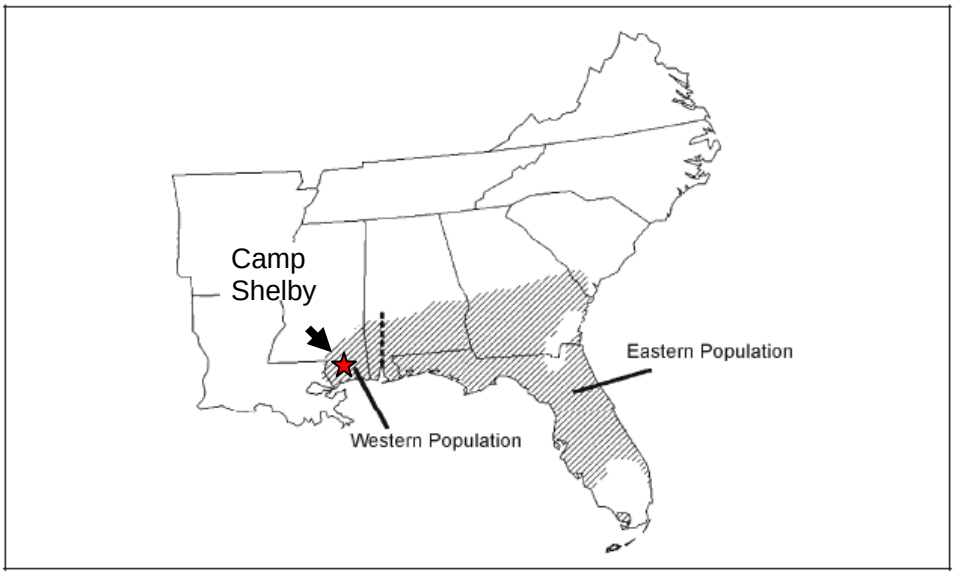

Figure 1. Approximate distribution of the gopher tortoise (modified from Wilson et al. [1997]).

This research will focus on gopher tortoises for three main reasons. First, because there is a concern about global declines in diversity and population abundances of herpatofauna in general [Alford and Richards 1999, Gibbons et al. 2000]. Thus, prevention of extinction of existing species is critical for maintaining biodiversity of these often overlooked, but ecologically important, taxa. Second, a large proportion of the remaining populations of gopher tortoises reside on DoD lands [Leslie et al. 1996]. Thus it is critical to develop strategies for preserving and maintaining these species on military facilities. Finally, gopher tortoise populations continue to decline in spite of conservation protection efforts [McCoy et al. 2006]. Thus, more information on the population biology and ecology of this species is needed to refine and improve management strategies.

Gopher tortoises inhabit upland ecosystems which have well-drained, deep sandy soils such as the longleaf pine, oak hammock, scrub pine flatwoods, and coastal dune ecosystems [Wilson et al. 1997]. Optimal habitat requirements include and open canopy (< 50 % canopy cover), abundant herbaceous understory, sandy soils with little silt (which facilitates burrowing), and sunny nesting sites. Closed canopy (>50% cover), silt/loam soils, and/or little to no ground vegetation are characteristics of poor habitats [Jones and Dorr 2004]. Mississippi longleaf pine soils differ from those in Florida, because they have a higher clay content, which may influence burrow architecture and contribute to the lower abundance and density in Mississippi than in the remainder of the range [Jones and Dorr 2004]. Tortoises dig burrows in sandy soils for shelter and protection from predators, may use more than one burrow, and will dig several burrows during their life [Wilson et al. 1997]. Most activity takes place close to the burrow, with tortoises rarely traveling farther than about 30 meters from their burrows. Gopher tortoises are a colonial species with several occupied and abandoned burrows being clustered in isolated patches of optimal habitat [Wilson et al. 1997]. Gopher tortoises are a keystone species instrumental in maintaining biodiversity in longleaf pine ecosystems, because the burrows and soil piles in front of them are foci of plant biodiversity and home to a variety of vertebrates and invertebrates [Lipps 1991, Wilson et al. 1997,]. Threatened and endangered animals that use gopher tortoise burrows include burrowing owls (*Speotyto cunicularia*), gopher frogs (*Rana capito*), and indigo and pine snakes (*Drymarchon corais* and *Pituophis melanoleucus,*

respectively) [Affenberg 1969, Affenberg and Iverson 1979, Jackson and Milstrey 1989, Deimer 1992]. Gopher tortoises are generally philopatric, and individuals commonly remain in their natal colony. They have a home range of ca. 0.4-1.1 ha [Eubanks et al. 2003], but will travel a distance up to 7.5 km [McRae et al. 1981]. However, the young have been known to disperse father following disturbance of their burrows [Eubanks et al. 2003]. Patterns of movement and dispersal are sex-biased, with males generally dispersing farther than females [Berry 1986]. Streams and rivers do not seem to be a dispersal barrier; however, roads may constitute a significant barrier to gene flow [Wallace and Anthony 2008]. Female tortoises may experience greater road-related mortality than males [Gibbs and Schriver 2002].

There have been several studies on the population genetics of gopher tortoises using mitochondrial or microsatellite markers [Osentoski and Lamb 1995, Schwartz and Karl 2000, Schwartz et al. 2003, Schwartz and Karl 2005, Schwartz and Karl 2008]. However, these studies have only examined population genetics over relatively large scales. Previous studies have failed to 1) use metapopulation-based population genetic, phylogeographic, Bayesian, maximum parsimony, maximum likelihood, multivariate ordination, and landscape genetic techniques as tools for assessing critical habitat needs for gopher tortoises *on DoD lands,* 2) examine *inter-colony* gene flow and genetic relationships within a population of gopher tortoises, 3) analyze population genetic structure of gopher tortoises in the *western part* of its range, where it is federally listed as endangered.

3.3 CONSERVATION GENETICS

Threatened and endangered species often exist as scattered metapopulations. This may contribute to their threatened status because 1) they are by nature specialized for living or reproducing in small, isolated patches, 2) human activities may disrupt connectivity among patches, and/or 3) landuse and development may fragment larger habitats into smaller patches that are less conducive to population sustainability and maintenance of biodiversity. Because gopher tortoises live in colonial burrows, which are often located in semi-isolated patches of optimal habitat, they fit the description of metapopulations. Metapopulations often exhibit extinction-recolonization and source-sink dynamics. An ecological “sink” is a population that is not able to sustain itself by recruitment from within the population, but may be maintained by recruitment from outside the population (for example, allopatric immigration) [Diffendorffer 1998]. “Source” populations are large and/or rapidly-growing populations in which the rate of emigration exceeds that of successful immigration [Diffendorffer 1998]. Source-sink dynamics are often vital for preventing permanent local extinctions and sustainability of the metapopulation as a whole.

Even with habitat fragmentation, metapopulations may persist in smaller, interconnected habitat patches if there are sufficient opportunities for dispersal among them [Franham et al. 2002]. In addition, metapopulations are able to persevere even if the local populations are extinction-prone [Franham et al. 2002]. Thus, even though gopher tortoises are somewhat sedentary, dispersal is an important component of their biology. However, anthropogenic activities may alter their distribution and dispersal patterns. They are sensitive to soil disturbance, and are less abundant or absent on disturbed soils in otherwise optimal habitat [Dale and Beyeler 2001]. Juveniles are especially sensitive to disturbance, and may move long distances if the burrow is disturbed [Perase and Crandall 2004]. In addition, habitat disturbance (for example, cultivation, mowing, fire suppression, development, or logging) or pollution may induce populations to be more “sink-like”, via curtailed reproduction and enhanced mortality. Genetic analyses could not only be used to identify potential source/sink dynamics, but, when

combined with GIS and landscape analyses, could identify potential dispersal corridors and foci of genetic biodiversity [Franham et al. 2002].

Relevant genetic analyses for endangered species management include metapopulation genetic investigations, landscape genetic approaches, and phylogeographic analyses [Perase and Crandall 2004]. Metapopulation analyses focus on dispersal and gene flow among populations, and are by their nature spatially explicit [Scribner and Chesser]. Landscape genetics analyzes spatial genetic data without the requirement of identifying discrete populations in advance [Manel et al. 2003]. As the name implies, it involves interpreting genetic variation and gene flow in the context of landscape structure. Phylogeography examines the geographic distribution of genotypes (or alleles) and their phylogenetic (evolutionary) relationships [Avise 1998]. Phylogeography can not only be used to delimit management units [Franham et al. 2002] (i.e., should all populations within a DoD facility be managed as one unit, or should management decisions be made on a site-specific basis?), but can help identify foci of genetic diversity, patterns of dispersal, and source/sink dynamics. Phylogenetic techniques such as nested clade analysis [Templeton 1998] can distinguish between historical and contemporary effectors of gene flow and genetic diversity. Spatially-explicit maximum likelihood-based genetic analyses [Paekteau et al. 1995, Beerli and Felsenstein 2001] can identify asymmetric dispersal and gene flow (i.e., dispersal from population A to population B vs. B to A).

The final reason that assessment of gene flow is important is that respiratory infections have been implicated, at least in part, in the continued decline of gopher tortoise diversity, despite conservation measures [McCoy et al. 2006]. It has been suggested that assessing long-term and long-range dispersal routes, which could be done using estimate of gene flow, is essential for tracking the spread and transmission of the causative agents of this disease [Smith et al. 1998, McGlaughlin 1997]. Such information could be important for predicting and monitoring the spread of the disease and developing management strategies which incorporate respiratory zoonotic dynamics into the conservation plans and viability analyses.

Finally, because gopher tortoises are long-lived with low recruitment, one could make the argument that the time since the inception of Camp Shelby (100 years) is too short to observe anthropogenic-induced evolutionary changes. However, although evolution normally takes place over long periods of time, microevolutionary processes can take place in a little as one generation in the case of strong selection pressures (for example, mediated by acute lethality), genetic drift caused by bottlenecks or founder events, extinction-recolonization, or dramatic alterations in the patterns of gene flow. All of these processes can be envisioned as taking place due to anthropogenic disturbance. Thus, gopher tortoise population genetic structure can theoretically be affected by human landscape alterations and habitat disturbance. Also, historic patterns of landuse may be more highly correlated to patterns of genetic diversity than contemporary patterns of landuse, especially for this long-lived species. An argument has also been made that current anthropogenic practices may lead to future genetic repercussions for tortoises [Edwards et al. 2005].

4.0 MATERIALS AND METHODS

4.1 APPROACH

Spatially explicit population genetic and phylogeographic analyses were used to determine relative levels of genetic diversity (within and between populations) and gene flow in the context of geographical position within Camp Shelby. Gopher tortoises were captured from twenty colonies in and around Camp Shelby [SERDP Project SI-1395] (Fig 2). These colonies were captured from areas delimited by two habitat descriptors and three descriptors indicating

the level of military activity, as described in SERDP Project SI-1395. There were six of these habitat/land use groupings, as follows: Group 1 - high military activity, good habitat, Group 2- low military activity, good habitat, Group 3 – low military activity, poor habitat, Group 4 – no military activity, poor habitat, Group 5 – no military activity, good habitat, forested, Group 6 – no military activity, good habitat, grass or ruderal type (Table 1). The qualitative descriptors "good" and"poor" are relative to habitat preferences of gopher tortoises.

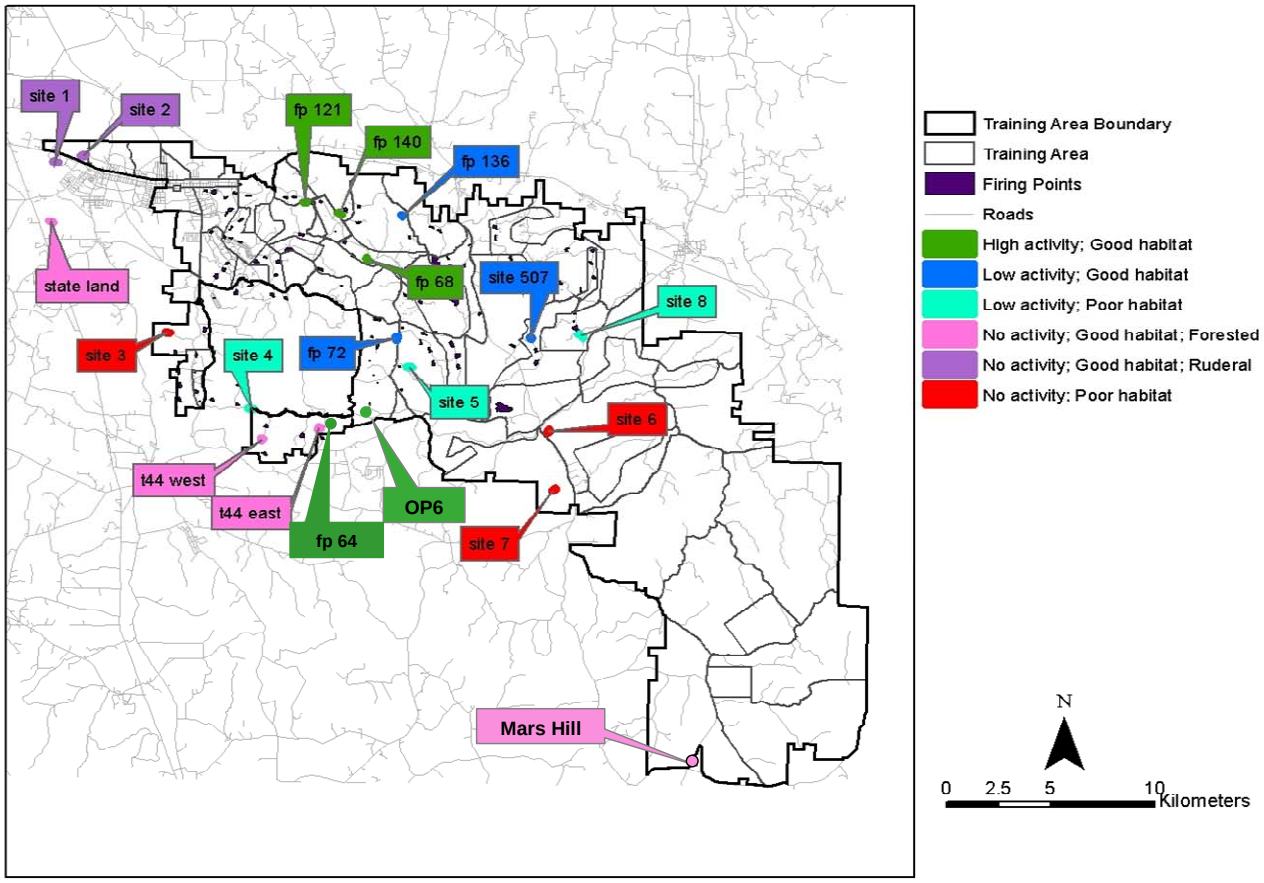

Figure 2. Map of Camp Shelby, showing sampling sites and military activity/ habitat quality

For the tortoises, two rapidly evolving molecular markers were employed: mitochondrial DNA (mtDNA) and nuclear microsatellite loci. The mtDNA was chosen because it provides fine-scale resolution of genetic differences [Raymond and Rousset 1995], should reflect gene flow between populations, and is maternally inherited. Microsatellite markers were used because they are highly polymorphic, possess large numbers of alleles, allow fine-scale spatiotemporal discrimination of within- and between-population genetic diversity, and are biparentally inherited [Chesser 1998]. Different results from maternally and biparentally inherited markers allow estimation of both male and female contributions to population dynamics [Chesser 1998]. Thus, microsatellites and mtDNA were essential for evaluating how populations are affected by habitat quality within and between populations. Also, using different markers with different mutation rates can provide inference about historic vs. recent genetic differentiation, which would contribute to delimitation of management units [Moran 2002]. The mtDNA and

microsatellites was amplified using Polymerase Chain Reaction (PCR) primers as described previously [Osentoski and Lamb 1995, Schwartz et al. 2003]. Because samples were not available for use as an out-group (in this case, the outgroup would have to be a gopher tortoise from the eastern population), unrooted trees were used for dendrogram analysis. Therefore, dendrograms only represent genetic similarity and do not imply common ancestry. Because genetic analyses are long-term, population-level phenomena, individual-level variables such as sex and age do not have significant effects on the genetic analysis (Hartl and Clark 1996).

Table 1. Characteristics of the sites on Camp Shelby from which gopher tortoises were collected.

Site	Group #	Level of military activity	Habitat quality	Sample size
FP 121	1	High	Good	12
FP 140	1	High	Good	7
FP 65	1	High	Good	4
FP 68	1	High	Good	8
OP6	1	High	Good	12
FP 136	2	Low	Good	4
FP 72	2	Low	Good	5
SITE 507	2	Low	Good	6
Site 3	3	Low	Poor	7
Site 4	3	Low	Poor	3
SITE 5	3	Low	Poor	7
Site 6	4	None	Poor	6
Site 7	4	None	Poor	8
Site 8	4	None	Poor	5
T44E	5	None	Good	12
T44W	5	None	Good	13
State lands	5	None	Good	7
Mars Hill	5	None	Good	10
Site 1	6	None	Good	9
Site 2	6	None	Good	7

4.2 TECHNICAL METHODOLOGIES

The amount of genetic diversity in mitochondrial DNA was analyzed in two different loci: a single-base sequence polymorphism in the cytochrome b gene (an enzyme in the electron transport chain) and the control region, a non-coding region that is the origin of DNA replication when the cell divides and replicates both nuclear and mitochondrial DNA. For mtDNA analyses, genetic diversity within populations was estimated as haplotype diversity [Nei 1987]. Genetic distance was calculated as the average number of nucleotide substitutions per site between populations [Nei 1987]. These statistics were estimated using the program DNASp22 [Rozas et al. 2003]. The mtDNA sequences were analyzed using neighbor-joining methodologies to assess relationships among haplotypes in each species [Swofford 1998]. Mitochondrial DNA was amplified using PCR primers. The sequences were determined using an automated sequencer, and the sequences were analyzed and aligned using Sequencher software (Gene Codes, Ann Arbor, MI). The mitochondrial DNA sequences were used to construct a phylogenetic tree of the haplotypes using the neighbor-joining algorithm.

For microsatellite analyses, genetic diversity was determined as average heterozygosity. Genetic distance was calculated according to Nei [1987]. Wright's fixation indices F_{ST} were

used to assess the amount of differentiation between-populations, respectively. Microsatellite markers were amplified using PCR, and allelic size variants were separated using an automated DNA sequencer. Collection of data and determination of allelic assignments were performed with the software program Genotyper. Genetic diversity and gene flow parameters were calculated using the software programs Microsat [Minch et al. 1996] and Genepop [Raymond and Rosset 1995].

The DNA sequences of mtDNA and the microsatellites were used to construct dendrogramsof the haplotypes. These dendrograms are based on DNA sequence similarities between haplotypes and can be visualized as a so-called "tree". These trees were constructed from genetic distances using the neighbor-joining algorithm. The genetic distances for microsatellites were based on average relatedness among individuals in different colonies, based upon the percentage of shared alleles. For cytochrome b sequences, the genetic distances were based upon average similarity of DNA sequence among individuals in different colonies. For the control region, genetic distances were based upon the relative proportion of individuals with each mitochondrial genotype in each colony.

5.0 RESULTS AND ACCOMPLISHMENTS

5.1 TECHNICAL PROGRESS

5.1.1 Go/No Go criteria

Before the analysis was initiated, the following Go/No Go criteria were devised. These were used to assess progress after the preliminary analyses to determine if there was justification to proceed with further analyses, and are as follows:

1. It must be possible to differentiate among populations using genetic markers. If the amount of gene flow is so high, or if the amount of genetic variation in these markers is so low, that these populations appear to be genetically homogeneous, then this project cannot proceed. (At least for that particular marker).
2. The markers must be polymorphic (genetically variable). If they are not, then these markers cannot be used. If no markers are variable, this project cannot proceed.
3. There must be enough genetic variability within and among populations to determine phylogenetic relationships between individuals and among populations.
4. There must be evidence of gene flow among populations, i.e., the populations cannot be totally genetically distinct.

5.1.2 Tasks and Milestones

Task 1. Determination of gopher tortoise genetic diversity and gene flow using mitochondrial

Milestone 1.1 Preliminary examination of incomplete mtDNA data set to determine if mitochondrial DNA is suitable for determining population genetics in gopher tortoises

Markers were analyzed for the mitochondrial DNA control region from 30 individual tortoises, and cytochrome b markers for 12 tortoises. It was found that there were 10 haplotypes for these 30 individuals, and two haplotypes for the cytochrome b primers. Using best

professional judgment, it was determined that this would be enough genetic variation to meet the Go/No Go criteria. This task was completed on 04/2007.

Milestone 1.2 Final analysis of Fst, migration, genetic diversity, and genetic distance data for mtDNA in gopher tortoises

A total of 130 individuals were analyzed with mitochondrial DNA cytochrome b locus. A second mitochondrial DNA locus, the control region, was less successful. Because of technical difficulties in PCR- amplifying this locus, only about 45 individuals could be analyzed. It is currently unknown why it was very difficult to amplify this particular locus. Data were analyzed using computer programs to determine patterns of gene flow and genetic diversity. These analyses were completed on 6/2007. Results are presented below.

Task 2. Determination of gopher tortoise genetic diversity and gene flow using microsatellites

Milestone 2.1 Preliminary examination of incomplete microsatellite data set to determine if microsatellites are suitable for determining population genetics in gopher tortoises

A total of 7 microsatellite loci were examined in approximately 100 individuals. Preliminary examination of these data reveals that 3 loci were monomorphic, but the remainder were polymorphic with 2-3 alleles per populations. Based on this best professional judgment, it was determined that microsatellite markers would have enough genetic diversity to be suitable for meeting the specific Go/No Go Criteria.

Milestone 2.2 Final analysis of Fst, migration, genetic diversity, and genetic distance data for microsatellite data in gopher tortoises

A total of 144 individuals were analyzed with 12 microsatellite markers. It was determined that seven were polymorphic, and five others were monomorphic. Data were analyzed using computer programs to determine patterns of gene flow and genetic diversity. These analyses were completed on 12/2007. Results are presented below.

5.2 EXPERIMENTAL RESULTS AND DISCUSSION

5.2.1 Specific Aim # 1: Testing if the populations are spatially structured

Table 2 reports Fst values for each pair-wise comparison for microsatellite DNA. This statistic is basically a ratio of the amount of genetic diversity between sites to the amount of diversity within sites. If the Fst is not statistically significantly different from 0, then this does not support the hypothesis that the two populations being compared are genetically distinct. Population genetic theory states that if the Fst is between 0 and 0.05, then there is a small amount of genetic distinctiveness between populations. If it is between 0.05 and 0.15, then there is a moderate amount of distinctiveness. If it is greater than 0.15, then there is a large amount of distinction between populations (Hartl and Clark 1996). It can be seen from the Table 2 that many of the Fst comparisons are not statistically significant ("NS"). However, this is not surprising, given the small number of samples (Table 1). However, there are a number of significant Fst values, most of which indicate moderate differentiation among populations. The data in Table 2 suggests that Site 2 68 and 121 are genetically distinct from all other populations. There is scant information in the literature as to the Fst values between gopher tortoise colonies

Table 2. Fst values for site-by-site comparisons for microsatellite DNA.

	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	FP 65	FP 68	FP 72	FP 121	FP 136	FP 140	FP 507	OP6	State Land	Mars Hill	T44W	T44E
Site 1	NS	NS	NS	NS	NS	NS	NS	0.08	0.06	NS	0.05	NS	NS	NS	NS	NS	NS	NS	NS
Site 2		0.12	NS	NS	NS	NS	0.12	0.12	0.07	NS	0.08	NS	0.13	NS	0.07	0.07	NS	0.05	0.05
Site 3			NS	NS	NS	NS	NS	NS	0.08	NS	0.06	NS	NS	0.17	NS	NS	NS	NS	NS
Site 4				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Site 5					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Site 6						NS	NS	NS	NS	NS	0.05	NS	NS	0.05	NS	0.04	NS	NS	NS
Site 7							NS	NS	0.06	NS	0.06	NS	NS	NS	NS	NS	NS	NS	NS
Site 8								NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
FP64									NS	NS	NS	NS	NS	NS	0.10	NS	NS	NS	NS
FP68										NS	NS	0.05	0.07	0.09	0.09	0.06	NS	0.05	0.06
FP73											0.04	NS	NS	NS	NS	NS	NS	NS	NS
FP121												NS	0.05	0.08	0.08	0.06	NS	0.03	0.03
FP136													NS	NS	NS	NS	NS	NS	NS
FP 140														0.12	NS	NS	NS	NS	NS
Site 507															0.07	NS	NS	NS	0.04
OP6																NS	NS	NS	NS
State Land																	NS	NS	NS
Mars Hill																		NS	NS
T44W																			NS
T44E																			NS

on such a small scale in other areas of the USA, so interpretation of these data in a larger context is difficult. However, other studies that examine the eastern population of gopher tortoises, as well as African tortoises have found less genetic differentiation between colonies on a much larger scale (> 50 km; Schwartz and Karl 2005, Paquette et al. 2007) This suggests that small-scale genetic structuring in tortoises from Camp Shelby is much greater than in other tortoises. These data are sufficient to satisfy specific Go/No Go Criteria #1 and #2.

5.2.2 Specific Aim #2: Testing if genetic diversity and gene flow is affected by habitat and military usage.

Figure 3 shows the amount of heterozygosity (a measure of genetic diversity) in each of the sampling sites, as measured by microsatellite DNA polymorphisms. Figure 3A illustrates differences in the number of tortoises captured at each colony. This is a reflection of the relative size of the population at each colony. According to these data, the no military, good habitat (forested) sites and the high military, good habitat sites had the highest number of tortoises per colony. Figure 3B is the overall heterozygosity, and data indicate that there is variation from site to site but not much difference among sites. However, genetic diversity estimates are greatly affected by the number of samples, so Figure 3C normalizes genetic diversity by sample size. The microsatellite DNA indicated that the colonies without military activity tended to have the least amount of genetic diversity, and colonies with military activity tended to have more genetic diversity. These levels of genetic diversity are similar to those found in desert tortoises (*G. agasazzi*; Edwards et al. 2004).

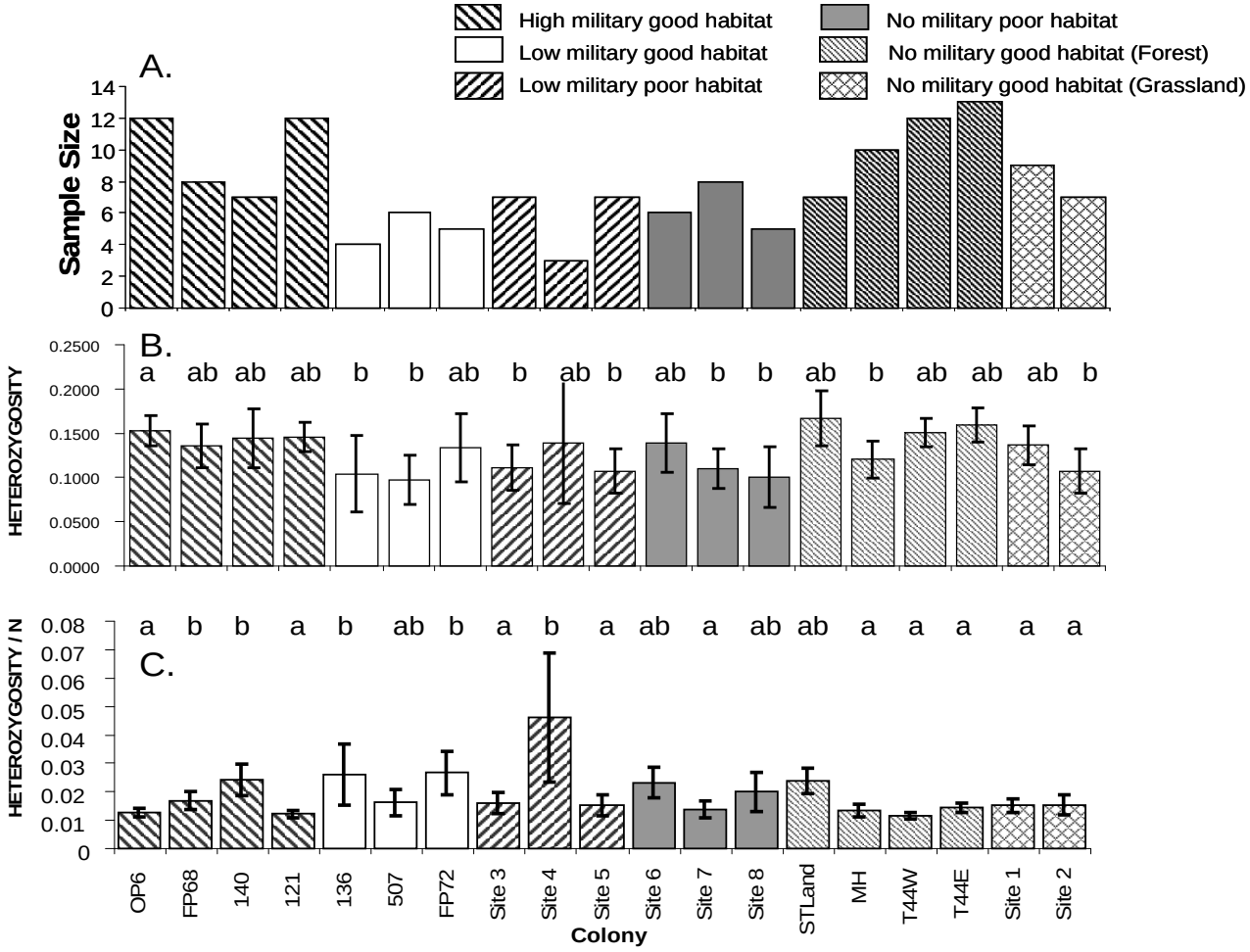

Figure 3. Microsatellite genetic diversity (heterozygosity) of gopher tortoises from 20 sampling sites on Camp Shelby. A. Number of tortoises collected, B. Heterozygosity, C Heterozygosity value divided by sample size. Error bars are 95% confidence limits. Bars labeled with different letters are statistically significantly different ($p < 0.05$, t-test with Bonferroni correction).

The mitochondrial sequences show a slightly different pattern of diversity (nucleotide diversity). Figure 4 illustrates the amount of genetic diversity calculated using the cytochrome b gene. Sites with no diversity lack bars on Fig 4. Figure 4A are the sample sizes for each colony, Figure 4B is the overall diversity, and Figure 4C is the diversity normalized for sample size. Figure 4B shows that colonies in the poor habitats tended to have the most diversity, whether they were in sites with no or low military activity, and the sites with good habitats tended to have lower diversity. For the good habitat sites, there were no clear distinctions among sites with high, low, or no military activity.

The data for the level of genetic diversity for the control regions are shown in Figure 5B. Figure 5C is the level of genetic diversity in the control regions normalized for sample size. These data indicate that the no military, good habitat sites had the lowest diversity, and the colonies with high military activity had the highest levels of genetic diversity, regardless of military activity (Figure 5C).

Gene flow among groups can also be reflected in the phylogenies, or "Gene trees", which graphically represent genetic relatedness and gene flow among groups. The tree based upon

microsatellite markers is shown in Figure 6A, the one based on the cytochrome b gene is shown in Figure 6B, and the one base on the control region is shown in Figure 6C. For comparison, a tree that was constructed based upon geographic distances is shown in fig 6D.

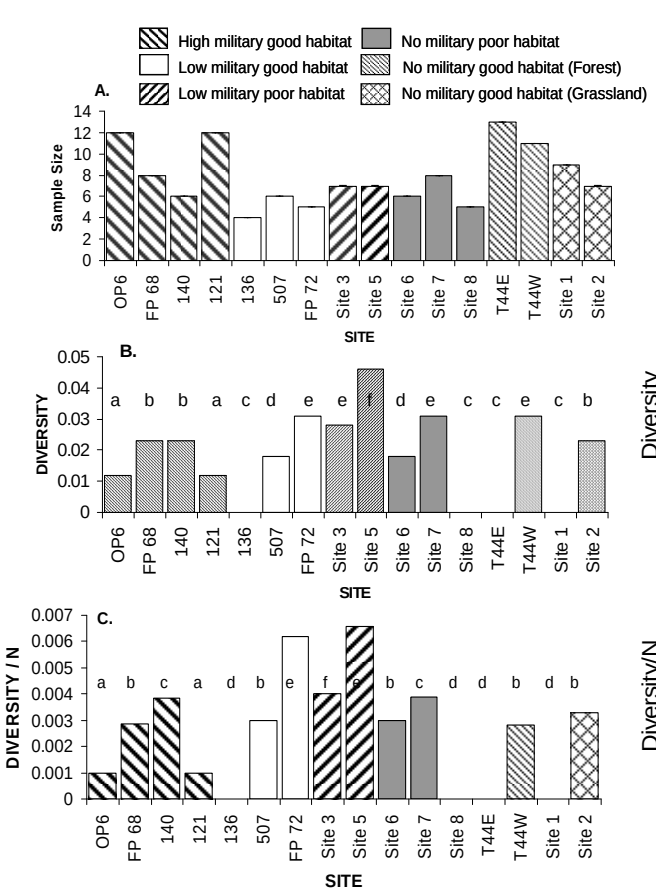

Figure 4. Number of tortoises collected (A), cytochrome b gene mitochondrial diversity (B) and mitochondrial diversity divided by sample size (C) for gopher tortoises at sites from Camp Shelby. Bars labeled with different letters are statistically significantly different (p< 0.05, t-test with Bonferroni correction).

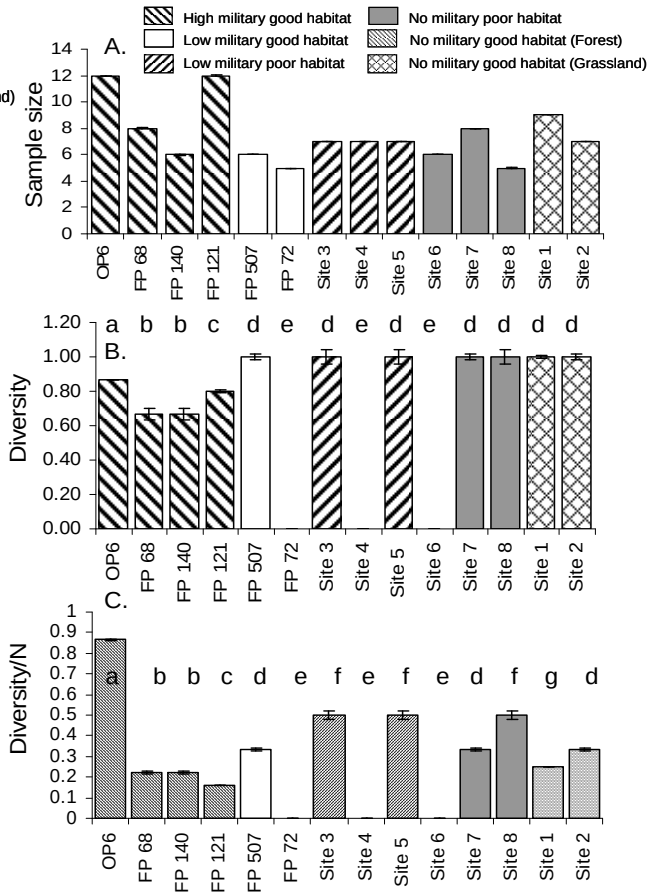

Figure 5. Number of tortoises collected (A), Control region mitochondrial diversity (B) and mitochondrial diversity divided by sample size (C) for gopher tortoises at sites from Camp Shelby. Bars labeled with different letters are statistically significantly different (p< 0.05, t-test with Bonferroni correction).

The tree represented in 6D is one that would be expected if genetic distance was completely correlated ($R^2 = 1.00$) to geographic distance (i.e., an idealized isolation by distance tree). The patterns of gene flow are what would be expected on the basis of geographic distance. However, for the microsatellite and mitochondrial trees, gene flow seems to be different than what would be expected on the basis of geographic distance. In particular, it would be expected that Site 1, Site 2, and the State Land site (sites with no military activity) would cluster together, given their geographic proximity (Figure 1). The same is true for the sites T44E and T44W. However, these sites are more closely related to the impacted sites (based upon patterns of clustering) than that are to each other. This suggests that gene flow among colonies is affected by factors other than geographic distance, possibly military activity. These data are sufficient to satisfy specific Go/No Go Criteria #1 – 4.

5.2.3 Specific Aim # 3 Testing if genetic distance is related to geographic distance

Correlation between geographic distances and genetic distances was testing using a Mantel test. The highest correlation coefficient was for microsatellite DNA markers 0.046, with a p = 0.46, and there were no correlations that were statistically significant (data not shown). These data indicated little, if any, correlation of geographic distance to genetic distance, as is predicted by the "isolation by distance" model of gene flow. This fact is also borne out by comparing Fig 5D (tree of geographic distances among sites) with Figure 5A-C (tree of microsatellite and mitochondrial DNA distances). This may be due to anthropogenic influences of gopher tortoises on Camp Shelby.

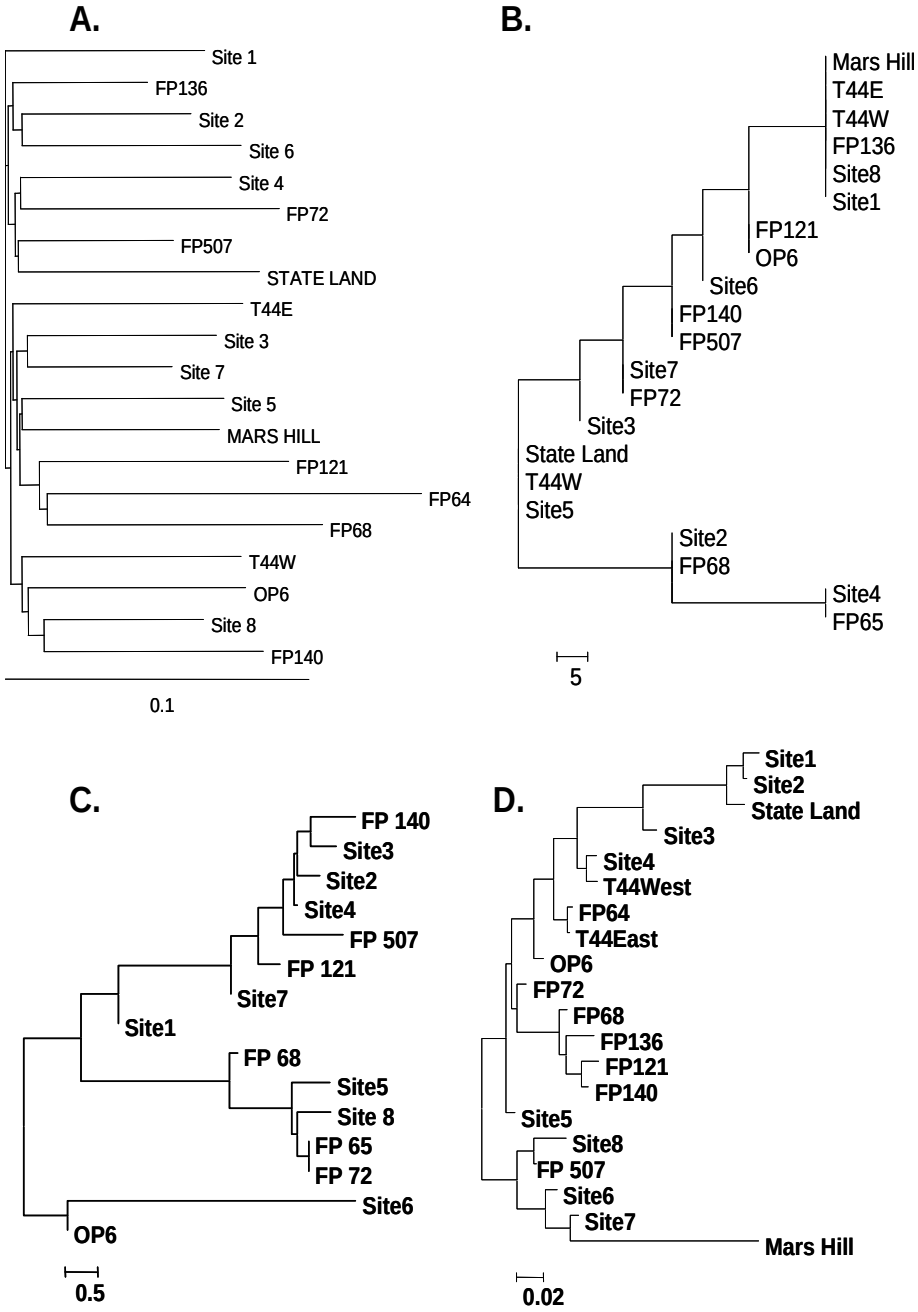

Figure 6. Phylogenetic trees representing genetic relationships among colonies of gopher tortoise populations. The trees were constructed using the neighbor-joining algorithm with microsatellite genetic distances. A) Tree using microsatellite-based genetic distances. B) Tree using mitochondrial cytochrome b genetic distances. C) Tree using mitochondrial Control region distances. D) Tree using geographic distance.

6.0 CONCLUDING SUMMARY

The data show that the relationship between gene flow, genetic diversity, geographic distance, habitat condition, and level of military activity is a complex one. Whether or not the data supported the original hypotheses was another story. Is the population on Camp Shelby spatially structured? Yes, surprisingly so. Significant Fst could be found even when the sample sizes were 12 or less. Is the genetic diversity and gene flow affected by habitat and land use? The data suggest that this may be the case, although there is much more to the story that needs to be discovered. Also, genetic distance does not seem to correlate with geographic distance. More detailed analyses including GIS-based landuse/landcover analyses, population demographics, and ecological analysis of habitat/movement/demographic interactions would be fruitful in dissecting out the mechanisms for the observed patterns. Overall, though, the data indicate that there is enough genetic diversity in the tortoises on Camp Shelby to be of use in identifying parcels of land for preserving critical habitat for maintaining dispersal corridors and sources of genetic biodiversity, because the amount of genetic diversity within this population is great enough to meet all of the specific Go/No Go Criteria, specifically Criterion #5.

7.0 FUTURE STUDIES

Although the preliminary studies above show that there are some indications that landuse and habitat may affect genetic diversity and gene flow, there are more studies that need to be done. First, before one can definitively determine if the patterns of gene flow and genetic diversity have been altered by human activity, one has to know what the background patterns in genetic variation might be in the absence of military activity. Second, the genetic analyses performed above are only preliminary and much more detailed and sensitive studies will need to be carried out, since the sample sizes at the individual gopher tortoise colonies are so small. Third, the study described above only examined landuse and habitat quality on a qualitative scale. It is not known what aspect of military activity or "habitat quality" might affect gopher tortoise genetic diversity. Fourth, it is not known if the patterns seen in the above study are only specific to Camp Shelby, or if they can be applied to other military sites. For example, do gopher tortoises in DoD reservations in the eastern population show similar trends, or are they completely different. Therefore, the specific aims of future studies will be:

1) **To compare patterns or genetic diversity on a DoD (Camp Shelby, MS and Egling Air Force Base, FL) and DOE (Savannah River Site) facilities and vicinity for gopher tortoises from other areas.**
2) **To carry out more detailed analyses of genetic diversity using analyses with more discriminating power.**
3) **To perform spatially-explicit, GIS-based analyses on the effects of landuse and landscape on gene flow and genetic diversity, on and off Camp Shelby, Eglin Air Force Base, and Savannah River Site.**

7.1 Conceptual Model

Figure 7 illustrates the mechanisms whereby fragmentation of habitat, military disturbance, and habitat quality can interact to affect genetic diversity within populations and genetic differentiation (or genetic distance) between populations.

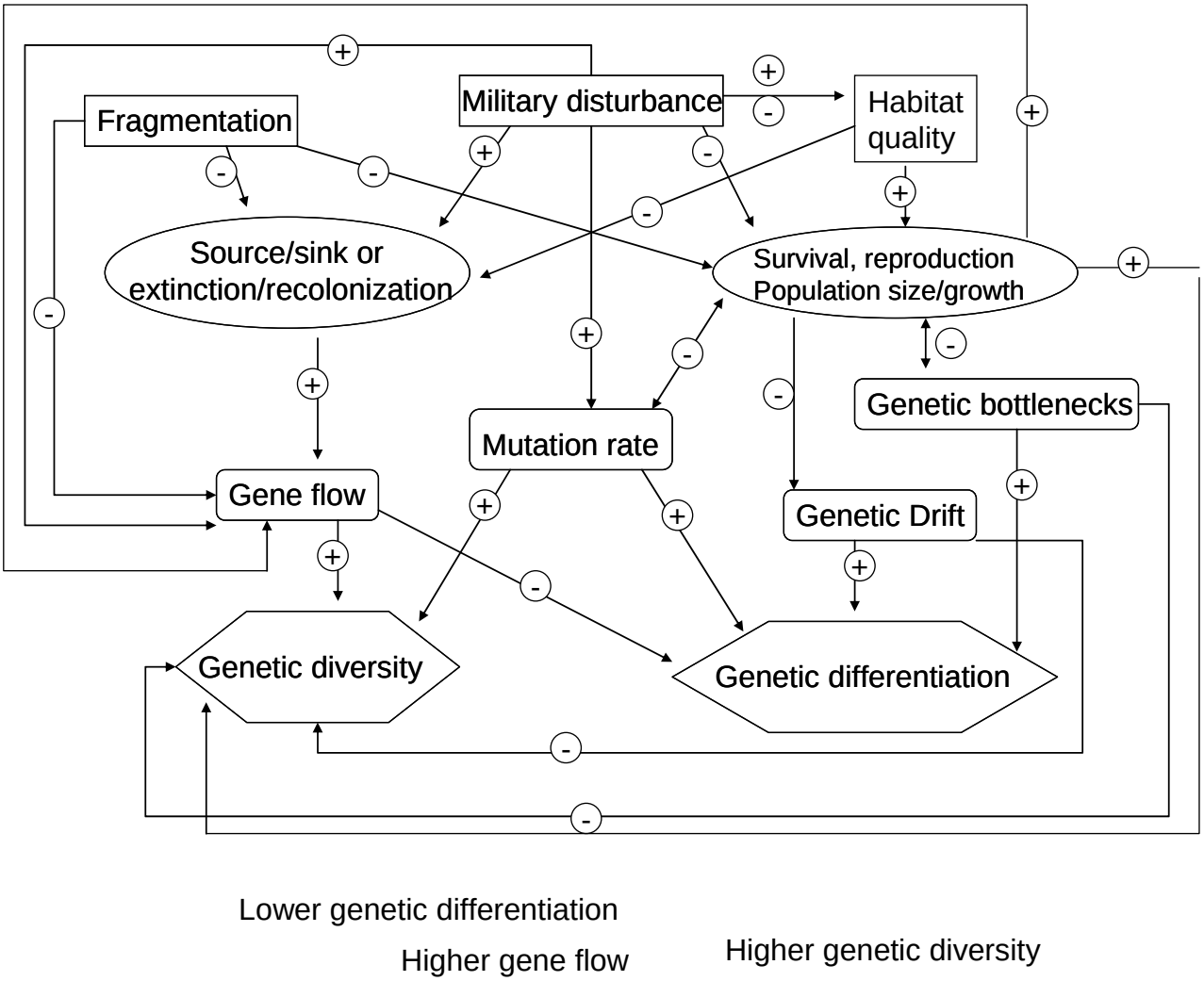

Figure 7. Conceptual model of the mechanisms whereby fragmentation of habitat, military disturbance, and habitat quality can interact to affect genetic diversity.

Habitat fragmentation can lead to reduced gene flow (via reduced amounts of dispersal), can reduce population size or growth rate, and can inhibit source/sink dynamics or ability to recolonize after a local extinction. Conversely, military disturbance can induce source/sink and extinction/recolonization dynamics. These types of dynamics will tent to increase gene flow among populations. Military disturbance can also induce more gene flow, for example if tortoises are more likely to leave a colony when it is disturbed. Military disturbance may also cause an increased mutation rate, if such activity results in contamination of the environment with mutatgenic chemicals. An increased mutation rate may increase genetic diversity (creation of new genotypes) and increase differentiation among populations. An increased mutational load in a population may suppress population growth, and conversely, larger populations tend to have lower mutational loads because they are less likely to accumulate mutations. Because mutational load reduced population size, and smaller populations tend to accumulate mutations faster, this can lead to a positive feedback loop known as a "mutational meltdown" [Franham et al. 2002]. Habitat quality will tend to reduce extinctions and prevent populations from becoming ecological sinks, and high quality habitats promote population growth. Military disturbance can affect

habitat qualtiy, by destroying habitat (negative interaction) or by creating habitat (for example, by converting a closed-canopy forest into an open savannah). This can indirectly affect population growth and size. Population growth, in turn, can stimulate gene flow, because more animals tend to disperse from large, rapidly-growing populations [Franham et al. 2002]. Larger populations also tend to have higher genetic diversity. Finally, population size and growth may influence two phenomena known as genetic bottlenecks and genetic drift. Genetic bottlenecks are rapid losses of genetic diversity caused by dramatic reduction in population size due to curtailed survival or reproduction. Loss of diversity due to bottlenecks can affect population growth [Franham et al. 2002]. Genetic drift is the gradual change in genetic makeup of a population due to stochastic processes, and may result in gradual erosion of genetic diversity due to random loss of genotypes from the population. Both genetic bottlenecks and drift tend ot increase genetic differentiation among populations and reduce genetic diveristy within populations [Franham et al. 2002]. However, genetic drift occurs more slowly in larger populations [Franham et al. 2002]. Thus, all of these factors may interact to affect genetic diversity within populations and genetic differentiation between them.

7.2 Research to Meet Specific Aims

7.2.1 Aim # 1: To compare patterns or genetic diversity on a DoD facility and vicinity for gopher tortoises from other areas.

This research will focus in part on colonies outside of Camp Shelby in Mississippi, in order to get an idea of the background genetic variation within and between colonies outside of Camp Shelby. Basically, in order to determine if military activities have an effect on genetic diversity, the "normal" patterns of genetic diversity must be ascertained. To do this, at least 15 colonies will be sampled and blood will be collected from the individuals in DeSoto National Forest and Paul B. Johnson State Park, and other areas in the vicinity. Analysis of genetic diversity and gene flow will be determined as described above and in section 7.3. For future phyogeographic analyses, gophers dendrograms will be rooted with outgroups. The tortoises from the eastern population (Florida) will be used as outgroups for the western population (Mississippi), and vice versa.

Furthermore, to determine the degree to which these approaches are applicable to other sites of interest to SERDP, these same approaches will be carried out at Eglin Air Force Base and at the DOE Savannah River Site. These studies will focus on at least 10 colonies residing on Eglin Air Force Base, FL, (part of the eastern population of gopher tortoises) and at least 10 gopher tortoise colonies in the vicinity of Eglin; similarly, this will be done for at least 10 colonies on the Savannah River Site and 10 colonies in the vicinity. Areas in the vicinity of Eglin Air Force Base will include those in Conuche National Forest and Pine Log State Forest. Areas in the vicinity of Savannah River Site will include Sumpter National Forest and Aiken State Park. Marshall Adams of Oak Ridge National Laboratory will be a collaborator on this part of the project, to capitalize on the relationships he has forged with personnel at Eglin. Dr. Theodorakis has also developed collaborative relationships with researchers that have ties to the Savannah River Ecology Laboratory. Comparisons of markers with different evolutionary and mutation rates, for example, the cytochrome b vs. microsatellite markers, would give an indication of patterns due to historical (cytochrome b) vs. more recent events (microsatellites).

7.2. 2 Aim # 2: To carry out more detailed analyses of genetic diversity using analyses with more discriminating power.

In order to increase the resolution and statistical power of the genetic analyses, more

microsatellite loci will be sampled. Because Luikart and Cornuet [1998] suggested that up to 20 polymorphic markers may be needed to detect recent population bottlenecks, I will attempt to identify at least nine new polymorphic markers, either by adapting them from published work with other chelonian species [Scwartz and Karl 2005], or by *de novo* cloning and sequencing.

In addition, more detailed analyses will also be carried out. Genetic methods could include three alternative approaches. The first would be to use Maximum Likelihood- or Bayesian Analysis-based assignment tests. If an individual is captured in an area outside the population to which it was "assigned", this would indicate a dispersal event, and the geographical distance from disperser to parent population could be determined. A second technique would be to use spatial autocorrelation, which is based upon the premise that the genotype of an individual is depended on the genotypes of nearby individuals, and that as individuals are further and further apart, they are less genetically similar, on average. The distance at which this relationship no longer holds is often taken as the maximum dispersal distance [Manel et al. 2003]. Multivariate autocorrelation analyses are now available for use with inter-individual genetic distances or genetic relatedness coefficients [Smouse and Peakall 1998]. A third alternative is to determine the genetic "neighborhood size". Theoretically, this is defined as $D\sigma^2$, where D is a function of population density and σ is a function of average per generation dispersal distance. Thus, if population densities are known from demographic studies, dispersal distances can be inferred [Slatkin and Barton 1989]. Efforts will are underway to determine population demographic parameters of gopher tortoises at Camp Shelby [SERDP Project SI-1395]. Use of more than one approach could be used as "weight of evidence" and confirmation that estimates of dispersal scale are accurate. Note that different techniques may give different estimates, but are almost always within an order of magnitude. This would be used to answer the question: should reserves, acquired land parcels, or managed areas be on the scale of m^2 or km^2, etc.

Genetic analyses will also incorporate data on fitness parameters, as was done by Adams et al. [SERDP Project SI-1395]. Gene flow (immigration/emigration ratios, per generation migration rate, Fst values among populations) and genetic diversity data will be integrated with population demographic and individual fitness components. Demographic and fitness components will be compared with genetic components to determine statistical relationships between genetic measures and fitness/demographic measures. This will be done using univariate and multivariate regression and correlation analyses. The genetic parameters to be used will include population genetic diversity estimates, gene flow/dispersal estimates, genetic relationships among populations, effective population size, neighborhood size, and immigration/emigration ratios. Demographic estimates of density will be combined with neighborhood size to estimate per-generation migration distancesThe hypothesis to be tested will be that with average fitness and population size/density will be directly related to genetic diversity and gene flow/dispersal distances.

7.2.3 Aim #3: To perform spatially-explicit, GIS-based analyses on the effects of landuse and landscape on gene flow and genetic diversity, on and off DoD and DOE facilities.

The effect of landscape on genetic diversity and gene flow will be tested by 1) comparing the level of genetic diversity (heterozygosity for microsatellites, nucleotide diversity for mitochondrial DNA,) between areas with different landscape properties; 2) determining if ratios of immigration/emigration differ between and among colonies on Camp Shelby, Eglin Air Force Base, Savannah River Site, and vicinity. Immigration and emigration rates will be determined by calculating asymmetric Nm values using MCML analysis (dispersal from population A to

pop. B vs. B to A), determining asymmetric dispersal using assignment tests (for example, number of individuals collected from population A and assigned to population B vs. number collected from pop. B and assigned to A), and by determination of the direction of dispersal by analysis of dendrograms; 3) comparing genetic distances between colonies residing in areas with different landscape characteristics. An example of this would be to compare average genetic distance between colonies on the base, average distance between colonies off-base, and average distance between on- and off-base colonies.

Landscape variables will include landuse categories, level of anthropogenic activities, landscape features, and soil type. Landuse categories will be closed-canopy forest (≥ 50% canopy cover), savannah/open forest (< 50% canopy cover), grassland, farmland, livestock pasture, developed land (urban, suburban, industrial, etc.), wetlands, and non-developed but disturbed lands (for example, clear-cut forests, mowed fields, city parks, golf courses, cemeteries, etc.). Level of military activity will be categorized into high, low, or no military activity categories. High activity areas classified as either a) highly developed land (for example, administrative or residential areas on the base) or b) non-developed, high impact areas such as firing ranges or sites used for training exercises. Low impact areas will be non-developed lands that have been anthropogenically modified, but are not used for military training directly (for example. areas adjacent to firing ranges, the area surrounding the airstrip on Camp Shelby, etc.). "No activity" sites will be sites that are not used for activities related to the mission of the facility, such as areas on the base designated as tortoise reserves or areas in the vicinity of the base. Landscape features will include roads, fencelines, streams, lakes, and airstrips. Soil type will be determined from maps downloaded from the USDA Natural Resource Conservation Service website. Landuse types and landscape features will be obtained from maps acquired from the US Geological Survey, USDA, the SIUE Department of Geography, Map Quest, Google Maps, and Google Earth.

There will be two catogories of landscape measurements. The first will be within-colony landscape metrics. For this metric, the area of the colony will be defined as a circle with a diameter of 670 m (the average size of a gopher tortoise colony [Eubanks et al. 2003]). The various landscape descriptors will be recorded inside this circle, and if different categories are present (for example, more than one soil type, landuse type, canopy type, etc.) the percentage of area covered by each landuse category will be measured using the program ArcView. The second landscape metric will be inter-colony landscape structure. This metric will be made by constructing straight-line transects between colonies from maps or aerial photographs, and recording quantitative measures of landscape variables along the transect, such as percent forest, developed land, number of roads along the transect, etc.

7.3 Applicability to identify the most ecologically important land parcels on and in the vicinity of DoD installations

Camp Shelby and Eglin Air Force Base are surrounded by privately-held properties and National Forests. Impacts of protecting TES species on military lands may be offset by identifying critical TES habitat on lands surrounding the DoD facilities. Such habitat on private land may be purchased by the Department of Defense, the Army, or the Air Force to offset losses in habitat on the bases due to military activities. For lands on the National Forests, these habitats may be the focus of preservation (for example, establishment of Wilderness Areas) or management for gopher tortoise habitat. Such vegetation management actions may include prescribed fire, midstory control, and intermediate forest stand thinning [Jones and Dorr 2004]

The Fsts (or Rsts for microsatellites) will be used to assess the degree of genetic differentiation among populations: the higher the Fst, the more similar the populations are genetically. Increased Fst implies increased gene flow and dispersal between populations. The Fsts will be calculated between populations on the bases and in the surrounding area. The areas for prioritization for purchase (private land) or management (National Forests) will be based upon the Fst values between the base and non-base populations: the non-base populations with highest Fst compared to base populations will be priority for purchase, preservation, or habitat management. Newer techniques that delimit population boundaries without a priori designation of populations can also be used to determine if the populations on- and off-site are separate populations [see Manel et al. 2003 for discussion].

Asymmetric dispersal patterns, as revealed by asymmetric Nm's (determined using MCML) and assignment tests would also be useful in managing endangered herpetofauna on DoD sites. First, they could be used to identify major sources of dispersal or "sources" on DoD sites and in the surrounding areas (populations with a higher emigration/ immigration ratio). These would be sites that would have the highest priority for purchase, preservation, or habitat management. In addition, the emigration/immigration ratio is expected to increase with population growth, density, and habitat quality [Diffendorfer 1998]. Thus emigration/immigration ratios are not only useful for identifying source/sink dynamics; they are also useful for determining population growth/density and habitat quality. Therefore, populations with the highest emigration/immigration ratios will be the highest priority for land purchase or habitat preservation/management. Offsite land parcels will also be prioritized according the sites with the highest genetic diversity (so-called "foci of biodiversity" [Semlitsch and Bodie 1998]. Parcels of land for purchase or management would also be identified if they were identified as corridors of dispersal: i.e., if these parcels were situated between populations that were connected by gene flow.

To this end, integration of population genetic, landscape, and GIS analysis (referred to as "landscape genetics" [Manel et al. 2003]) would be highly valuable. In this regard, determining the empirical relationships between genetic attributes (genetic diversity and gene flow) and landscape/landuse attributes would be invaluable. The landscape attributes would include habitat characteristics (soil type, dominant vegetation, vegetation density, open or closed forest canopy, presence and density of herbaceous understory, wetland or waterway density and area), landscape morphometrics (dominance, contagion, fractal dimension, elevation, slope, surface topography), and anthropogenic landuse (agricultural areas, urban areas, pine plantations, pasture, of roadways, harvested forests, managed woodlands or grasslands). The empirical genetic/landscape relationships will allow future management activities to use remote sensing and landscape analysis to identify parcels of land with the potential of maximizing genetic biodiversity and gene flow of endangered species while minimizing cost to the military and impact of conservation on military activities on the bases. The benefits of this include a cost-effective method of categorizing habitats and identifying potential agricultural and other anthropogenic impacts without the need to intrusively sample a large number of endangered species populations. Rather, landscape, GIS and remote sensing would be used to prioritize sites as potential foci of genetic biodiversity and gene flow. Then the genetic analysis could focus on these limited number of high-priority sites.

Landscape genetic analysis can also be used to prioritize sites most in need of restoration, management, or remediation. For example, landscape genetics could identify landscape attributes that may restrict or prevent gene flow. Now say there was a competing landuse conflict whereby a large tract of land was needed for military activities, but it was also a focus of

genetic biodiversity. Suppose also that outcome of the genetic landscape analysis also indicated that the loss of this large patch could be offset by preserving smaller, interconnected patches. The GIS approach could identify is the habitat and landscape between the patches was suitable for gene flow, and, if not, how these corridors could be restored, remediated, or managed to promote gene flow. Alternatively, if the military activities result in degradation of habitat quality and subsequent genetic biodiversity, landscape analysis could be used to identify off-site parcels of degraded land that could be restored or managed to improve their potential for maintenance of genetic diversity and dispersal. In this way, there would be no net loss of genetically valuable habitat.

Once populations have been ranked as to their level of genetic diversity and emigration/immigration potential, this will provide guidance as to which lands should be targeted for purchase or prioritized for management or preservation. However, the question would still remain: what is the spatial scale for land purchase, management, or preserve establishment. This could be addressed either demographic or genetic methods, or a combination of both. Demographic methods would use mark-recapture and tracking techniques to determine home range sizes and dispersal distances. Home range sizes and dispersal distances could then be used by resource managers to determine the size of land parcels to be purchased or the scale at which to manage tracts of forested land. For example, home range sizes of gopher tortoises have also been previously estimated [McRae et al. 1981, Eubanks et al. 2003].

Genetic methods may be used when demographic approaches are impractical or infeasible. For example, marking or radiotracking gopher tortoises may not be practical due to their low dispersal rates. In addition, mark-recapture and radiotelemetry studies would be impractical for determining long-term trends in long-range, infrequent dispersal [Moran 2002]. Genetic methods could include three alternative approaches. The first would be to use Maximum Likelihood- or Bayesian Analysis-based assignment tests. If an individual is captured in an area outside the population to which it was “assigned”, this would indicate a dispersal event, and the geographical distance from disperser to parent population could be determined. A second technique would be to use spatial autocorrelation, which is based upon the premise that the genotype of an individual is depended on the genotypes of nearby individuals, and that as individuals are further and further apart, they are less genetically similar, on average. The distance at which this relationship no longer holds is often taken as the maximum dispersal distance [Manel et al. 2003]. Multivariate autocorrelation analyses are now available for use with inter-individual genetic distances or genetic relatedness coefficients [Smouse and Peakall 1998]. A third alternative is to determine the genetic “neighborhood size”.

Finally, phylogenetic analyses will also be used to assist land managers in identifying parcels of land off-site that are valuable for managing TES. The dendrograms constructed from the mtDNA haplotypes will be used to assess evolutionary relationships among the individuals and populations analyzed. This will serve several purposes. First, it has been recommended that conservation management strategies preserve as many distinguishable genetic lineages (major “branches” on the “tree”). This would preserve as much genetic diversity as possible, maximize evolutionary plasticity and sustainability in the face of stochastic and anthropogenic environmental changes in the future, and minimize the potential for inbreeding in these populations. When the phylogenic “tree” is interpreted in light of geospatial distributions, it will allow determination of which off-site populations should be the focus of land acquisition or national forest management, for example, by preventing the acquisition of lands on which populations with extremely similar evolutionary histories reside. Second, phylogeographic approaches such as nested clade analysis can be used to distinguish genetic similarity due to

shared evolutionary history from genetic similarity due to recent gene flow [Templeton 1998]. This would be instrumental both for prioritizing land units based upon gene flow and for prioritizing them based upon evolutionary history. Third, the individual-based dendrograms may be used to infer patterns of dispersal as described above. Again, when interpreted in light of geographic distribution of individuals, this would provide information on the scale of dispersal distances and thus, the scale of land units to be purchased or managed. Because directional dispersal can be identified, this technique can also be used to determine relative rates of immigration and emigration. Fourth, the number of individuals at the terminal branches of the tree is correlated with rate of population growth and density. Thus, the dendrogram can be used to prioritize land units on the basis of number of tip haplotypes. Fifth and finally, phylogeographic and population genetic approaches can determine historical patterns of dispersal that may no longer be viable for the surviving tortoises. For example, genetic analyses may indicate that a large amount of dispersal has historically occurred among colonies, but that recent habitat modifications have occurred that may curtail present or future dispersal. In order to restore dispersal corridors among colonies, which enhances long-term sustainability, parcels of land between genetically-interconnected colonies may be purchased and restored to a state that facilitates gopher tortoise dispersal.

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APPENDIX A – Supporting Data

Table A1 – Microsatellite allele frequencies[1] for gopher tortoises collected from various colonies on and around Camp Shelby, MS.

			Colony (sampling site)																			
Locus[2]	Allele	Size[3] (base pairs)	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	FP 65	FP68	FP 72	FP 121	FP 136	FP 140	FP 507	OP 6	State Land	Mars Hill	T44 W	T44 E
GOAG 3	1	369									25											
	2	375	100	100	100	100	100	100	100	100	75	100	100	100	100	100	100	100	100	100	100	100
GOAG 4	1	117		7	7																	
	2	123	94	93	93	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	3	129	6																			
GOAG 5	1	259	6																14	6		5
	2	262	94	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	86	94	100	91
	3	265																				5
GOAG 6	1	400	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
GP 15	1	222												8								
	2	224	33	7	14	17	21	8	13	40	13	6	20	21	25	42	33	38	43	17	32	32
	4	228	39	43	79	50	43	33	69	30	75	50	40	50	50	25	33	25	43	50	32	50
	5	232	11	36	7		21	42	13	20		13	20	8	25	17	8	29		17	23	14
	6	238				17	7							4			8	4	7	6	9	
	7	240	17	14		17	7	17	6	10		31	20	4		8	17	4	7	11	5	5
	8	258									13			4								
GP 19	1	255	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
GP 26	1	361	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
GP 30	1	212																				5
	2	214															8			6		5
	3	224					14				25	38		25				4	7	6	9	
	4	226	100	93	100	83	86	92	100	100	63	56	80	58	100	92	92	96	93	89	91	91
	5	228										6	10	4								
	6	230		7		17		8			13		10	13		8						
GP 55	1	272	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
GP 61	1	206	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
GP 81	1	408	94	100	83	83	100	83	88	100	63	86	70	100	100	83	100	79	79	100	85	86
	2	410	6		17	17		17	13		38	14	30			17		21	21		15	14
GP 96	1	140	69	86	50	83	50	67	71	40	63	64	80	58	75	40	92	58	79	56	58	59
	2	146	31	14	50	17	50	33	29	60	38	36	20	42	25	60	8	42	21	44	42	41

[1]Percentage of individuals in the colony in which this allele is found. I.e. a frequency of 100 means that this allele is found in all individuals in that particular colony.
[2]The names of the loci are arbitrary and are only used for reference. The numbers do not have any particular meaning. "Goag" stands *for Gopherus agasazzi* (the desert tortoise, for which the PCR primers were originally developed) and "GP" stands for *G. polyphemus*.
[3]Size of the PCR product that was amplified from this locus.

Table A2 – Genetic distance[1] between pairs of gopher tortoise colonies on and around Camp Shelby, MS, as determined by microsatellite genotypes

	Colony																		
	Site1	Site2	Site3	Site4	Site 5	Site6	Site7	Site8	FP 65	FP 68	FP 72	FP 121	FP 136	FP 140	FP 507	OP6	State Land	Mars Hill	T44W
Site2	0.127																		
Site3	0.136	0.133																	
Site4	0.142	0.131	0.143																
Site 5	0.138	0.133	0.133	0.151															
Site6	0.145	0.128	0.146	0.152	0.145														
Site7	0.122	0.111	0.11	0.124	0.125	0.13													
Site8	0.127	0.131	0.127	0.15	0.122	0.139	0.123												
FP 65	0.209	0.203	0.184	0.199	0.205	0.213	0.178	0.21											
FP 68	0.173	0.161	0.168	0.171	0.165	0.175	0.154	0.17	0.214										
FP 72	0.158	0.146	0.161	0.155	0.167	0.159	0.142	0.163	0.214	0.184									
FP 121	0.161	0.153	0.155	0.163	0.151	0.17	0.145	0.151	0.21	0.173	0.183								
FP 136	0.113	0.101	0.114	0.122	0.117	0.122	0.097	0.11	0.187	0.154	0.139	0.138							
FP 140	0.148	0.157	0.149	0.167	0.146	0.159	0.145	0.125	0.221	0.19	0.176	0.172	0.134						
FP 507	0.117	0.107	0.134	0.121	0.13	0.133	0.109	0.124	0.2	0.159	0.141	0.147	0.097	0.146					
OP6	0.144	0.142	0.148	0.158	0.146	0.149	0.137	0.132	0.217	0.185	0.166	0.172	0.126	0.147	0.136				
State Land	0.147	0.148	0.153	0.152	0.16	0.166	0.137	0.151	0.213	0.188	0.168	0.177	0.131	0.166	0.131	0.16			
Mars Hill	0.136	0.13	0.131	0.147	0.132	0.145	0.121	0.126	0.204	0.167	0.165	0.154	0.115	0.151	0.127	0.149	0.157		
T44W	0.144	0.141	0.145	0.155	0.143	0.151	0.134	0.133	0.212	0.179	0.167	0.166	0.125	0.15	0.134	0.147	0.16	0.146	
T44E	0.144	0.143	0.139	0.155	0.146	0.156	0.13	0.136	0.209	0.182	0.169	0.168	0.125	0.155	0.136	0.154	0.159	0.145	0.153

[1]Genetic distance is inversely related to the average genetic similarity between two colonies. I.e., the greater the genetic distance, the less genetic similarity. This distance was computed on the basis of average relatedness between individuals in different colonies.

Table A3 – Frequency of fifteen cytochrome b haplotypes[1] in gopher tortoise colonies on and around Camp Shelby, MS.

	Frequency[2]	
Colony	Haplotype 1	Haplotype 2
Site 1	100	
Site 2	25	75
Site 3	60	40
Site 4		100
Site 5	50	50
Site 6	80	20
Site 7	67	33
Site 8	100	
FP 121	87.5	12.5
FP 136	100	
FP 140	75	25
FP 172	100	
FP 507	75	25
FP 65		100
FP 68	25	75
FP 72	67	33
OP6	87.5	12.5
Mars Hill	100	
State Land	50	50
T44E	100	
T44W	50	50

[1]A haplotype is a mitochondrial genotype. A given haplotype may be found in more than one tortoise (i.e., different tortoises may share the same haplotype). Cytochrome b is an enzyme of the electron transport chain.

[2] Percentage of individuals in the colony in which this haplotype is found. I.e. a frequency of 100 means that this allele is found in all individuals in that particular colony.

Table A4 – Genetic distance[1] between pairs of gopher tortoise colonies on and around Camp Shelby, MS, as determined by cytochrome b haplotypes[2]

	Distance from colony																			
To colony	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	FP 121	FP 136	FP 140	FP 172	FP 507	FP 65	FP 68	FP 72	OP6	Mars Hill	State Land	T44E
Site 1																				
Site 2	75																			
Site 3	40	35																		
Site 4	100	25	60																	
Site 5	50	25	10	50																
Site 6	20	55	20	80	30															
Site 7	33	42	7	67	17	13														
Site 8	0	75	40	100	50	20	33													
FP 121	12.5	62.5	27.5	87.5	37.5	7.5	20.5	12.5												
FP 136	0	75	40	100	50	20	33	0	12.5											
FP 140	25	50	15	75	25	5	8	25	12.5	25										
FP 172	0	75	40	100	50	20	33	0	12.5	0	25									
FP 507	25	50	15	75	25	5	8	25	12.5	25	0	25								
FP 65	100	25	60	0	50	80	67	100	87.5	100	75	100	75							
FP 68	75	0	35	25	25	55	42	75	62.5	75	50	75	50	25						
FP 72	33	42	7	67	17	13	0	33	20.5	33	8	33	8	67	42					
OP6	12.5	62.5	27.5	87.5	37.5	7.5	20.5	12.5	0	12.5	12.5	12.5	12.5	87.5	62.5	20.5				
Mars Hill	0	75	40	100	50	20	33	0	12.5	0	25	0	25	100	75	33	12.5			
State Land	50	25	10	50	0	30	17	50	37.5	50	25	50	25	50	25	17	37.5	50		
T44E	0	75	40	100	50	20	33	0	12.5	0	25	0	25	100	75	33	12.5	0	50	
T44W	50	25	10	50	0	30	17	50	37.5	50	25	50	25	50	25	17	37.5	50	0	50

[1]Genetic distance is inversely related to the average genetic similarity between two colonies. I.e., the greater the genetic distance, the less genetic similarity. This distance was computed on the basis of the frequency of control region haplotypes in each colony.

[2] A haplotype is a mitochondrial genotype. Cytochrome b is an enzyme of the electron transport chain.

Table A5 – The information[1] for the various haplotypes[2] used for analysis of the control region[3] mitochondrial DNA in gopher tortoises in and around camp Shelby.

Haplotype	Total # TAs[4]	Total # TAAs[5]	Total # repeats[6]	Total # bases[7]	#TATAATAAs[8]	#TATAAs[9]	#TAs[10]
H1	12	19	31	81	9	1	2
H2	11	17	28	73	8	1	2
H3	11	18	29	76	9	0	2
H4	12	20	32	84	10	0	2
H5	11	19	30	79	9	1	1
H6	11	18	29	76	9	2	1
H7	12	21	33	87	10	1	1
H8	7	13	20	53	6	1	0
H9	8	13	21	55	6	1	1
H10	8	15	23	61	7	1	0
H11	9	17	26	69	8	1	0
H12	10	19	29	77	9	1	0
H13	11	21	32	85	10	1	0
H14	10	20	30	80	9	0	0
H15	11	22	33	88	10	0	0

[1] Based upon a TATAATAA repeat amplified from the 3' end of the mitochondrial DNA light strand. The DNA sequence TATAATAA was repeated several times in this locus, with genetic diversity being represented by different numbers of TA or TAA subunits

[2] A haplotype is a mitochondrial genotype. Each haplotype has a particular number of TA and TAA repeat units. A given haplotype may be found in more than one tortoise (i.e., different tortoises may share the same haplotype).

[3] The control region is the origin of replication of the mitochondrial genome.

[4] Total number of TA repeat units in the haplotype.

[5] Total number of TAA repeat units

[6] Total number of repeating units, either TA or TAA.

[7] Total number of nucleotide bases in the haplotype.

[8] Number of times in the haplotype DNA sequence that the sequence "TATAATAA" occurs uninterrupted.

[9] Number of repeat units that consist of a TATAA, i.e., number of repeat units that lack a second TAA component.

[10] Number of repeat units that lack any TAA component.

Table A6 – Frequency of fifteen mitochondrial control region haplotypes[1] in gopher tortoise colonies on and around Camp Shelby, MS.

	Frequency[2] of Haplotype:														
Colony	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15
Site 1		25	25		25							25			
Site 2			33		33									33	
Site 3												50	50		
Site 4														100	
Site 5							50				50				
Site 6				100											
Site 7					33					33		33			
Site 8					50		50								
FP65	50											50			
FP 68					67							33			
FP 72					100										
FP 121	20										40	40			
FP 140					67										33
FP 507					33		33		33						
OP6			10		30	10		10			10	30			

[1] A haplotype is a mitochondrial genotype. Each haplotype has a particular number of TA and TAA repeat units. A given haplotype may be found in more than one tortoise (i.e., different tortoises may share the same haplotype). The control region is the origin of replication of the mitochondrial genome.
[2] Percentage of individuals in the colony in which this haplotype is found. I.e. a frequency of 100 means that this allele is found in all individuals in that particular colony.

Table A7 – Genetic distance[1] between pairs of gopher tortoise colonies on and around Camp Shelby, MS, as determined by mitochondrial control region haplotypes[2]

	Distance from colony													
To Colony	FP 121	FP 140	FP 507	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	FP 65	FP 136	FP 68	FP 72
FP 140	2.015													
FP 507	1.322	2.197												
Site 1	1.386	1.792	1.792											
Site 2	1.322	1.099	1.504	1.099										
Site 3	1.609	1.099	1.792	1.386	1.099									
Site 4	0.916	1.099	1.099	0.693	0.405	0.693								
Site 5	2.303	10	10	2.079	10	10	10							
Site 6	10	10	10	1.386	10	10	10	10						
Site 7	1.099	1.504	1.504	0.875	0.811	1.099	0.405	1.792	10					
FP 65	1.609	10	10	1.386	10	10	10	0.693	10	1.099				
FP 136	2.303	10	10	2.079	10	10	10	1.386	10	1.792	0.693			
FP 68	2.708	2.197	10	2.485	10	1.792	10	1.792	10	2.197	1.099	1.792		
FP 72	1.609	10	10	1.386	10	10	10	0.693	10	1.099	0	0.693	1.099	
OP6	2.12	2.708	3.401	1.743	2.303	2.996	2.303	1.609	2.303	1.609	0.916	1.609	2.015	0.916

[1]Genetic distance is inversely related to the average genetic similarity between two colonies. I.e.., the greater the genetic distance, the less genetic similarity. This distance was computed on the basis of the frequency of control region haplotypes in each colony.

[2] The control region is the origin of replication of the mitochondrial genome. Each haplotype has a particular number of TA and TAA repeat units. A given haplotype may be found in more than one tortoise (i.e., different tortoises may share the same haplotype). A haplotype is a mitochondrial genotype.